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Development of an Efficient *In Vivo* System (P_{junc}-TpaseIS*1223*) for Random Transposon Mutagenesis of *Lactobacillus casei*

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The random transposon mutagenesis system P_{junc}-TpaseIS*1223* is composed of plasmids pVI129, expressing IS*1223* transposase, and pVI110, a suicide transposon plasmid carrying the P_{junc} sequence, the substrate of the IS*1223* transposase. This system is particularly efficient in *Lactobacillus casei*, as more than 10,000 stable, random mutants were routinely obtained via electroporation.

Lactic acid bacteria (LAB) are widely used in food fermentations, as well as for their probiotic properties. *Lactobacilli delbrueckii* subsp. *bulgaricus* and *Lactobacillus casei* have been shown to provide beneficial effects to the immune system (29, 35). However, due to the lack of reliable tools such as a random mutagenesis system to perform global reverse genetics, the overall mechanisms underlying their probiotic effects are poorly understood.

Neither the Gram-positive transposition systems based on transposon delivery by a suicide or a thermosensitive vector (19, 27, 34, 36) nor *in vitro* transposon mutagenesis using Tn*5*-based transposons (17) is adapted to all species of LAB, due to low transformation efficiencies or unwanted stability of the transposon delivery vector (23).

IS*3* sequences are surrounded by imperfect inverted repeats (IR). They carry two consecutive and partially overlapping open reading frames, *orfA* and *orfB*, which encode a transposase. IS*3* sequences undergo a "cut-and-paste" transposition mechanism that occurs by generating a covalently closed circular transposition intermediate, which promotes transposase induction resulting from the generation of a strong promoter named P_{junc}. The P_{junc} promoter corresponds to abutted IRR (inverted repeat right) and IRL (inverted repeat left) sequences as a result of insertion sequence circularization and constitutes an efficient transposition substrate (13, 14). Here, we report the construction of a novel in *trans* transposition procedure, named the P_{junc}-TpaseIS*1223* system and dedicated to *in vivo* random mutagenesis in LAB, and its application for random mutagenesis in *L. casei*. It is based on IS*1223*, a member of the IS*3* family from *Lactobacillus johnsonii* (39) that transposes efficiently in *Lactobacillus delbrueckii* subsp. *bulgaricus* (31, 39). This system is composed of two plasmids: pVI129, carrying the IS*1223* transposase gene, and pVI110, a suicide transposon plasmid carrying the P_{junc} sequence, the substrate of the IS*1223* transposase.

Construction of the P_{junc}-TpaseIS*1223* system and validation in *Escherichia coli*. Plasmid pVI116 was constructed as described in Fig. 1 and its legend to provide the transposase of IS*1223* expressed under the control of the *L. delbrueckii* subsp. *bulgaricus* P_{hlbA} promoter (9). Plasmid pVI115 was constructed from pVI162 (see Table 1 for details of construction) to provide the transposition substrate corresponding to an abutted IRR-IRL junction of IS*1223* separated by 3 base pairs, named P_{junc} (Fig. 1A). It replicates only in the TG1 RepA strain of *Escherichia coli* (18). Plasmid pVI116 (P_{hlbA}-Tpase$_{IS1223}$) and the control plasmids, pGB2 and pVI113 (Tpase$_{IS1223}$), were electroporated into *E. coli* TG1 as previously described (12). The resulting strains were electroporated with identical amounts (100 ng) of pVI115, as a nonreplicative source of the P_{junc}, or pVI119 (Table 1), as a P_{junc}-less nonreplicative control, and pGEMT, as a replicative plasmid. Cells were directly plated on LB agar plates supplemented with chloramphenicol (10 μg/ml) or ampicillin (50 μg/ml). Plates were incubated for 20 h at 37°C, and colonies were counted to score for integration or transformation. Since pVI115 cannot replicate in these *E. coli* strains, the resulting Cm^r transformants were considered pVI115 chromosomal integrants. The integration efficiency obtained with pVI115 in the absence of Tpase$_{IS1223}$ was very low ($\sim 10^{-8}$) compared to that of the strain carrying a Tpase$_{IS1223}$ without a cloned promoter ($\sim 10^{-6}$), as well as that obtained with Tpase$_{IS1223}$ fused to the P_{hlbA} promoter (up to 10^{-3}). In all strains, the integration efficiency of pVI119 was very close to the background level observed in the absence of identified promoter (10^{-8} to 10^{-7}). These results clearly show that Tpase$_{IS1223}$ triggers pVI115 integration using the P_{junc} substrate in *trans* and that the P_{hlbA} promoter drastically enhances the expression of Tpase$_{IS1223}$ in *E. coli*. The transposon as developed in this work mimics the double-strand DNA intermediate and integrates as a nonreplicative element in the target sequence (Fig. 1C). The target sites of 19 integrants were determined by direct sequencing of genomic DNA (GATC Biotech) using the primer OLB215 (Table 2), which targets one transposon extremity (Fig. 1C). Fourteen insertions had occurred in different putative open reading frames; three were located in noncoding regions and two in repetitive extragenic palindromic (REP) sequences (data not shown). To confirm ran-

Received 20 February 2012 Accepted 3 May 2012

Published ahead of print 18 May 2012

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doi:10.1128/AEM.00531-12

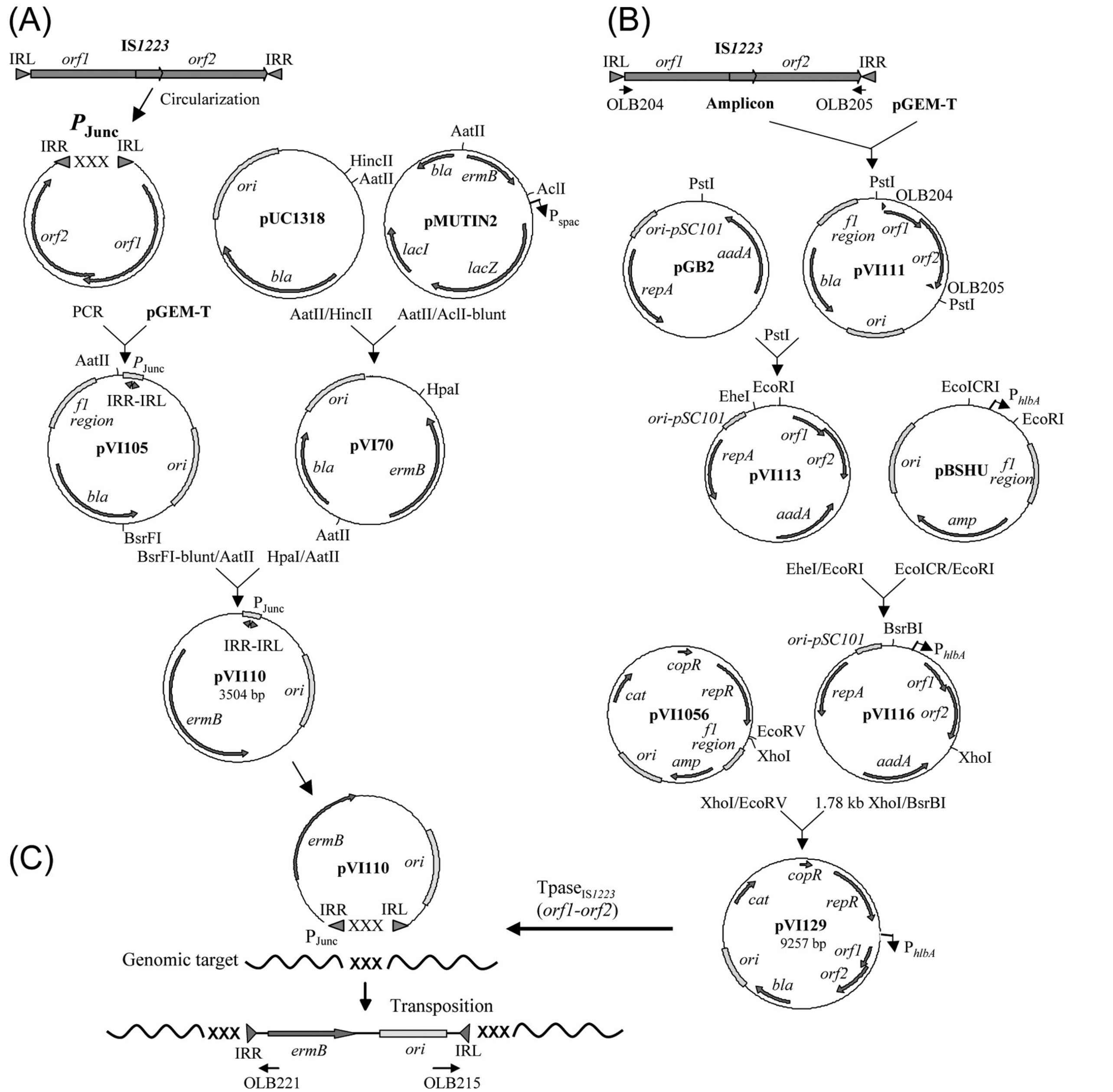

FIG 1 Diagram of plasmid construction. (A) Construction of the suicide transposon vector pVI110. (B) Construction of the transposase IS*1223* (*orf1*-*orf2*)-delivering vector pVII129. *orf1*-*orf2* are expressed under the promoter of the *L. delbrueckii* subsp. *bulgaricus hblA* gene (P_{hlbA}). The characteristics of each plasmid are indicated in Table 1. The genes *bla*, *aadA*, *cat*, and *ermB* encode resistance to ampicillin, spectinomycin, chloramphenicol, and erythromycin, respectively. (C) Map of integration of pVI110 into genomic DNA by the action of Tpase IS*1223* on P_{junc}, with indication of primers OLB221 and OLB215 (Table 2) used for sequencing. XXX corresponds to the 3 to 4 base pairs duplicated during integration of pVI110 in the genomic target. Plasmids are not drawn to scale.

domness of integration and saturation of the chromosome by pVI115, a pVI115-mutagenized Lac-positive (Lac$^+$) *E. coli* strain culture was diluted and spread onto LB medium with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) to screen Lac-negative (Lac$^-$) mutants. Of the 10 Lac$^-$ clones analyzed, none was redundant, strongly supporting the randomness of integration and saturation of the *E. coli* chromosome by pVI115. Altogether, these results validated the fact that $Tpase_{IS1223}$ is active in *E. coli* and efficiently recognizes P_{junc} as a substrate leading to random transposition.

P_{junc}-TpaseIS$_{1223}$ *in vivo* transposon mutagenesis in *L. delbrueckii* subsp. *bulgaricus* and in *L. casei*. The highly efficient P_{junc}-$Tpase_{IS1223}$ transposition system was adapted to LAB, namely, *L. delbrueckii* subsp. *bulgaricus* and *L. casei*. P_{hlbA}-$Tpase_{IS1223}$ was cloned in the *E. coli*–Gram-positive bacterium shuttle vector, pVI1056, to give pVI129 (Fig. 1B and Table 1), a plasmid providing the $Tpase_{IS1223}$. Plasmid pVI129 possesses the pIP501 replication origin, which is thermosensitive in several Gram-positive bacteria (4, 15, 21, 26, 30), including *L. delbrueckii* subsp. *bulgaricus* (31). This property allows the efficient elimination of the

TABLE 1 Bacterial strains and plasmids

Strain or plasmid	Relevant markers, phenotypes, characteristics, and construction	Reference or source
Strains		
E. coli		
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F′ [*traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	16
TG1*repA*	TG1 derivative with *repA* gene integrated into the chromosome	18
TG1pGB2	TG1 plus pGB2	This work
TG1pVI116	TG1 plus pVI116	This work
TG1pVI113	TG1 plus pVI113	This work
L. delbrueckii subsp. *bulgaricus*		
VI104	ATCC 11842 type strain	32
LBpVI129	VI104 carrying pVI129	This work
LBpVI1056	VI104 carrying pVI1056	This work
L. casei		
LC334	ATCC 334 type strain	Collection Institut Pasteur, France
LCpVI129	*L. casei* ATCC 334 carrying pVI129	This work
LCpVI1056	*L. casei* ATCC 334 carrying pVI1056	This work
Plasmids		
For construction of pVI115 and pVI116 and experiments with *E. coli*		
pBluescriptSK$^-$	Ap^r, pBR322*ori*	1
pBSHU	Ap^r, pBluescriptSK$^-$ containing 317 bp of *L. delbrueckii* subsp. *bulgaricus hlbA* promoter region	9
pGB2	Sp^r, pSC101*ori*	10
pGEM-T	Ap^r, pBR322*ori*, f1*ori*, linear T-overhang vector	Promega
pJIM2242	Erm^r, pWV01ori	18
pVI42	Ap^r, pBluescriptSK$^-$ IS*1223* cloned at ClaI and EcoRI sites	31
pVI62	pVI42 with abutted P_{junc}, generated by cloning the two complementary oligonucleotides OLB187 and OLB188 between the ClaI site treated with exonuclease VII and the XhoI site of pVI42	This work
pVI105	Ap^r, pGEM-T with a 136-bp sequence containing P_{junc} amplified with OLB131 and OLB203 primers using pVI62 as a template	This work
pVI107	Cm^r, pGEM-T containing the *cat* gene from pACYC184	This work
pVI108	Em^r, pJIM2242 containing the P_{junc} SpHI-PstI fragment (185 bp) of pVI105	This work
pVI111	Ap^r, pGEM-T containing IS*1223*ΔIR	This work
pVI113	Sp^r, pGB2 containing IS*1223*ΔIR	This work
pVI115	Cm^r, pWV01*ori*Δ*repA*, P_{junc}, obtained by ligation of the SpHI-EcoRII (extremity filled in with the Klenow fragment) P_{junc}-containing fragment of pVI108 and the SpHI-HincII fragment (carrying the *cat* gene from pACYC184) of pVI107	This work
pVI116	Sp^r, pGB2 containing P_{hlbA}-IS*1223*ΔIR	This work
pVI119	Cm^r, pVI115ΔP_{junc}, obtained by self-ligating pVI115 digested with HincII-SchI	This work
pVI138	Em^r, *E. coli-L. delbrueckii* subsp. *bulgaricus* shuttle vector	This work
For construction of pVI110 and pVI129 and experiments with lactobacilli		
pGB3631	Em^r, pIP501 derivative	6
pGKV259	Cm^r, Gram$^-$/Gram$^+$ shuttle vector	38
pIP501	Em^r, Gram$^-$/Gram$^+$ shuttle vector, containing the replication origins from pBluescriptSK$^-$ for Gram$^-$ and from pIP501 for Gram$^+$, including the copy number-controlling *copR* gene	22
pMUTIN2	Ap^r Em^r, pBR322*ori*	37
pUC1318	Ap^r, pBR322*ori*	24
pVI70	Em^r, pUC1318 containing the *ermB* gene from pMUTIN2	This work
pVI110	Em^r, pBR322*ori*, P_{junc}	This work
pVI129	Ap^r Cm^r, pVI1056 containing P_{hlbA}-IS*1223*ΔIR	This work
pVI137	Em^r, pBR322*ori*	This work
pVI1052	Ap^r Em^r, pBR322*ori*, pIP501*ori*, obtained by ligation of pGB3631 and pBluescriptSK at EcoRI-BamHI sites	This work
pVI1056	Ap^r Cm^r, pBR322*ori*, pIP501*ori*, *cop*$^+$, low-copy-number replicative plasmid in *L. delbrueckii* subsp. *bulgaricus*, obtained by ligation of the XhoII fragment (extremities filled in with the Klenow fragment) containing the Cm resistance (*cat-86*) cassette fragment of pGKV259 and the Eco47III-XbaI (extremity filled in with the Klenow fragment) of pVI1052	This work

transposase-expressing plasmid so as to avoid further transposition events. The P_{junc} sequence was combined with the erythromycin resistance cassette *ermB* to generate pVI110 (Fig. 1A), the suicide transposon plasmid.

L. delbrueckii subsp. *bulgaricus* VI104 and *L. casei* LC334 cells were first transformed with pVI129 as previously described (2, 32), and the resulting strains, LBpVI129 and LCpVI129, respectively, were then electroporated with 4 μg of pVI110, an optimal amount determined by preliminary assays with different amounts of plasmid DNA (data not shown). The cells were directly plated on MRS agar plates supplemented with erythromycin (5 μg/ml), and the plates were incubated for 2 days at 42°C or 37°C for VI104 and LC334 strains, respectively, under static anaerobic growth conditions. The Em^r colonies obtained after transformation with suicide transposon pVI110 were considered genomic (chromosome or indigenous plasmid) integrants. The transposition efficiency was determined by the number of Em^r colonies obtained for 50 μl of electrocompetent cells with 1 μg of pVI110 plasmid. With LBpVI129, the number of integrants was estimated between 300 and 1,500 for $\sim 2 \times 10^8$ viable cells, while with LCpVI129, this number reached between 2,500 and 7,500 for $\sim 10^9$ viable cells in more than 10 independent experiments. These results demon-

TABLE 2 Primers

Primer use and name	Sequence (5′ to 3′)[a]	Target
Plasmid construction		
ERYF	GTTGATAGTGCAGTATCTTA	*erm*C
ERYR	CTTGCTCATAAGTAACGGTAC	*erm*C
IRLL-P_{junc}	GATCTTTATGTCTAACAATTATGAGGC	pVI62
IRLR-P_{junc}	AAGTGCCTCATAATTGTTAGACATAAAACGACTCCTGTAAAATACAG	pVI62
M13rev	AACAGCTATGACCATG	pVI62
OLB93	AATGTAGGAAAGAAAGCACC	pVI62
OLB131	ACGACTCCTGTAAAATACAG	M13-OLB93 amplicon
OLB187	TCGAATGTCTAACTTTTCTATGGCACTTC	Complementary to OLB188
OLB188	GAAGTGCCATAGAAAAGTTAGACAT	Complementary to OLB187
OLB203	AAAATACCTCATAATTATTAGATTTTATGTCTAACAATTATGAGGCAC	M13-OLB93 amplicon
OLB204	AAATCTGCAGTTATGAGGTATTTTTTTATGACC	IS*1223*
OLB205	ACATTTCTCGAGTTTTAAAGATTTGATAATACACG	IS*1223*
pVI110 target sequencing		
OLB215	ATGGCCGCGGGATTACGACTCC	pVI110
OLB221	AGCTATGCATCCAACGCGTTGGG	pVI110
Plasmid copy no. in *L. casei*		
LSEI0004F	ACCACCACAAGTTTGGAAGG	LSEI_0004
LSEI0004R	TCACGCTCTTGCTAATGTCC	LSEI_0004
LSEI0145F	CGAAACCGAGGACTTGTTG	LSEI_0145
LSEI0145R	AATGTGCGGGCTGAGAAC	LSEI_0145
LSEIA04F	ACTGGCACCAACGGATAGTC	LSEA_04 (pLSEIA)
LSEIA04R	GATGGCATTGAGACGACAGA	LSEA_04 (pLSEIA)
LSEIA13F	TTTGTTCGCTATCGGTTTCC	LSEIA_13 (pLSEIA)
LSEIA13R	AGTGGTTGATCGCACGACTA	LSEIA_13 (pLSEIA)

[a] Underlined bases indicate PstI restriction sites.

strate that P_{junc}-TpaseIS*1223* is functional and efficient in the two species. Negative controls were made using two other combinations of plasmids introduced successively: (i) pVI1056, as a $Tpase_{IS1223}$-nonexpressing vector, and pVI110 and (ii) pVI129 and pVI137, a P_{junc}-less plasmid. For the two strains, less than 10 Em^r colonies were obtained using these plasmid combinations. These last data show that the P_{junc} is not the substrate of a genomic indigenous putative transposase produced by *L. delbrueckii* subsp. *bulgaricus* or *L. casei* and that P_{junc} is strictly required for pVI110 integration in these two species.

Preliminary results (data not shown) revealed that the growth of *L. casei* was seriously affected at temperatures above 40°C, mak-

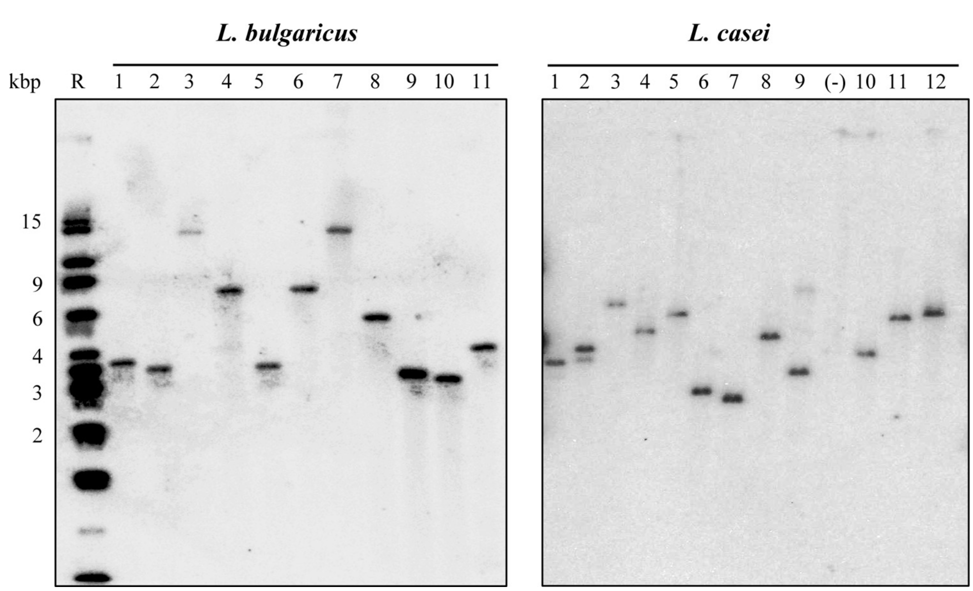

FIG 2 Diversity of pVI110 integration in *L. delbrueckii* subsp. *bulgaricus* and *L. casei*. Southern analysis of 11 *L. delbrueckii* subsp. *bulgaricus* integrants' DNA restricted by NogMIV and 12 *L. casei* integrants' DNA restricted by HindIII with a ^{32}P-labeled pVI110-*ermB* probe. R, Raoul molecular weight marker; (−), no DNA sample.

TABLE 3 Identification of target in independent *L. delbrueckii* subsp. *bulgaricus* and *L. casei* pVI110 integrants

Species and integrant[a]	pVI110 insertion site sequence[b]	Locus of pVI110 insertion[c]
L. delbrueckii subsp. *bulgaricus*		
Lb1	TTTCTTGGAATTAAAGCGCATAGTTA|AATCACTTCTTCTTTTTCTTCTTTTCTT	IGR Ldb2182/Ldb2183
Lb2	TAAAAAAGTCTCGCTGAAAAGCGGGA|CTTTTTTGCGCCTTTGACGTGATTTTACA	IGR Ldb1406/Ldb1407
Lb3	AGATCATTCTTCAAAAAGAGCTCCCG|GAATCCGGGAGCTCTTTTGCTTTAGTTAA	Ldb0913
Lb4	AAAACAATACGAAGCAAAAGCAAGAA|GAAAAAGCATGTTTGAAAAAACATGCTTT	IGR Ldb0494/ldb0501
Lb5	TGAGACCTATGTAAGAAGCTCAGGTC|CACAGGACCGAGCTTCTTTTGTGCTTTTA	IGR Ldb2110/Lbd2112
Lb6	CATAAGCAACAAAAAAGCAGTCATTC|CATCGATGACTGCTTTTCTGCTGCTGTGT	IGR Ldb0218/Ldb0219
Lb7	CGATAAAAAAGAAGGTCAGCGCGGCA|AAAAGCGCTGGCCTTTTTAAATTAGATTT	IGR Ldb2015/Ldb2020
Lb8	CAGAATTTAGAGCAAAGTAAAAGCCG|CTTTTCAGCGGCTTTTATTTTTTTCTTGT	IGR Ldb1491/Ldb1492
Lb9	GATGACAAAAACAGGCTGAGGCCTAT|TTTTATTTTGCCTTTTTTCTTCTTTTTTT	IGR Ldb1733/Ldb1734
Lb10	CAAATAGCAAAGAAAAAAACTAGCTGA|AAAATCAGCTAGTTTTTTCTTTTTCCCGT	IGR Ldb0968/Ldb0968
Lb11	ACAAAGCTTTAAAAAGCGCTACAGGA|CAACTTGCAGCGCTTTTTAGTTTTGTAAT	IGR Ldb0164/Ldb0165
Lb12	AAGCCGCCAACTACGGAATCTTGGAC|CTTGCCAAAAGCCCCGGTTACTTTTCCG	Ldb2064
Lb13	ATGTAGAAAAGAAAACGAAGCTGCTC|AAAGTGAGTAGCTTCGTTTTTTGCTATTA	IGR Ldb2034/Ldb2036
Lb14	TGTAACCTAAACTAATCCTTTTGGCA|ATTTTCCTGGGCTTTTTTTGCTAATTTTT	Ldb1636
Lb15	ACAAAAATCTTGCTTAACTAATTGCA|TTATATAACGGCTTTTTTGAATTTTGTTA	IGR Ldb2086/Ldb2087
Lb16	ATACAAGGAAAAAAAGAGCTCCAGAA|CTTGCTAAACGCTTCTGAAGCTCTTTCTT	IGR Ldb2086/Ldb2087
Lb17	AATCAAACGAAAAAGCTTCAGTAAAG|CAATACTGAAGCTTTTTTCATTGCTATTA	IGR Ldb2090/Ldb2091
Lb18	TAGCAATGAAAAAAGCTTCAGTATTG|CTTTACTGAAGCTTTTTCGTTTGATTCTA	IGR Ldb2090/Ldb2091
Lb19	ATACAAGGAAAAAAAGAGCTCCAGA|ACTTGCTAAACGCTTCTGAAGCTCTTTCTT	IGR Ldb2086/Ldb2087
Lb20	AAGAAAGAGCTTCAGAAGCGTTTAG|CAAGTTCTGGAGCTCTTTTTTTCCTTGTAT	IGR Ldb2086/Ldb2087
L. casei		
Lc1	CGCTGGCGGATTATGTGACACCGGAAA|ATGACTGGGAGCCGCTCAATTTTTCAG	LSEI_1278
Lc2	AAAAAAGCTCACGTTTTGCGACGTGAG|CTTTTTTGGTGCCGTCAGAACAAGTTA	IGR LSEI_1440/LSEI_1441
Lc3	AGTGAAGCTCCAGACCGTGAATTACAC|AACGGTGAAAAAACCATCAACGGTTCT	LSEI_1892
Lc4	GTCACCGATGACAGCGCCAAGCTTTTC|CGCGATTTGCCAAAAGATCAAACCGTC	LSEI_1979
Lc5	TACACTGATGTTGAGAGATCAACATCA|GTGTACAGCTCTTTTATTTTGGGCCTA	IGR LSEI_2050/LSEI_2051
Lc6	TTTTTGGTTAAGGGCTTTTAATTTAGC|TTGTTTTTCTAAGTTACTTTGCGACAT	IGR LSEI_A13/LSEI_A14
Lc7	CTTTGTGCTTATGCTGGGGATTGGAAT|TCTTAGACTGTTTTTTTCGTTTTTTAC	LSEI_0106
Lc8	TAAAAAGTGGCCCCGCGTAAATACTGC|AACGAGGCCACTTTTTATATTTATGGG	IGR LSEI_2579/LSEI_2580
Lc9	ACTCAGGTGATTTCACATAGCTCCATG|TTGCCTGAGAGCCTTTTAATTTAGGCA	LSEI_0797
Lc10	TGACCGGCAGGGTCATTGTCGGAGCCA|ACATAAATAGTGGCTGGCAATTGCCCT	LSEI_0548
Lc11	ATTCAAAAAAAGTTAAAAGACTTTGCT|AAACACAATCCAGAAATTAAGGCAAAA	IGR LSEI_A13/LSEI_A14
Lc12	TGGCCCTGCGTAATTTGACTTGAAACA|ACTGTTGGAAAGTTCTTTAATTTTTCT	LSEI_0374
Lc13	GTTGGCAGTCAGCAAGTCGCTTTAAAA|GCAGTCACCAATCAGAAAGACTATGAT	LSEI_2769
Lc14	GACGAAAAAACAAAGAAGGTATCAGCC|TAAACGCCGGTACCTTCTTTATTATCT	IGR LSEI_0637/LSEI_0638
Lc15	ATCAAAGATACTAAACAGCTTCTTAAG|AGATTTTAGACAGCTTCTAAACACCAT	IGR LSEI_0343/LSEI_0344
Lc16	TTCTTGCTCAACAAAAAAACCACCACG|AGGGTGGAAAAGTTTGGGGGAACTTTT	IGR LSEI_2568/LSEI_2569
Lc17	TTCAGGTGCAGCAAAAACAGTTTACCG|ATACGCAACTCGAAACTGCTACGAGTT	LSEI_2855
Lc18	CTGAACTCTTTGGCCTTGGAAAATCAG|ATAGGTAGTTTTGACGTTCTATTTCCT	LSEI_1966
Lc19	CCATAAGGAACACATGCACAATGCCCA|AAAAAGACCATTGCATTTGTGCGCCGA	IGR LSEI_1566/1567
Lc20	CGCGTTACTAAAAAGAAGCTATATCTG|ATGCACAGCATTCTGCTGGGCGCGATA	IGR LSEI_2333/LSEI_2334

[a] Lb and Lc indicate *L. delbrueckii* subsp. *bulgaricus* and *L. casei*, respectively, in integrant designations.
[b] Inverted repeat sequences are underlined, and vertical bars are pVI110 insertion sites.
[c] IGR, intergenic region.

ing the elimination of pVI129 at a high temperature undesirable. The segregational stability of pVI129 in *L. casei* at 37°C was estimated at 86% per generation as described by Heap et al. (20). Thus, the inherent pVI129 instability and the resulting loss of $\text{Tpase}_{\text{IS}\mathit{1223}}$ in *L. casei* mutants considerably limit the risk of genomic instability of the mutants.

Analysis of pVI110 integration in *L. delbrueckii* subsp. *bulgaricus* and *L. casei*. pVI110 insertion mutants of *L. delbrueckii* subsp. *bulgaricus* and *L. casei* were randomly selected. Mutant genomic DNA digested by NgoMIV for *L. delbrueckii* subsp. *bulgaricus* and by HindIII for *L. casei* was analyzed by Southern hybridization with a pVI110-specific probe generated by PCR amplification with primers ERYF and ERYR (Table 2). Plasmid pVI110 integrated in a single locus in each mutant, except for mutant 2 of *L. casei*, which presented two bands of different intensity, suggesting two distinct mutants in the sample (Fig. 2). Overall, the diversity of fragment sizes among the tested clones indicated that pVI110 had inserted randomly into both the *L. delbrueckii* subsp. *bulgaricus* and *L. casei* genomes. *L. casei* strain LC334 carries pLSEIA (GenBank accession number NC_008502.1), a 29-kbp indigenous plasmid. Since indigenous plasmids are often targets of preferential insertion, leading to a reduction in efficiency of random transposon mutagenesis in chromosomal targets (28), we determined the plasmid copy number (PCN) of pLSEIA by quantitative PCR (qPCR). Real-time PCRs were performed as previously described (25) from whole DNA of *L. casei* with primer pairs LSEI0004F-LSEI0004R and LSEI0145F-LSEI0145R (for the chromosome) and LSEIA04F-LSEIA04R and LSEIA13F-LSEIA13R (for pLSEIA) (Table 2). The PCN was determined using the following equation: PCN $= (Ec)^{C_{T}c}/(Ep)^{C_{T}p}$, considering different amplification efficiencies [$E = 10^{(-1/\text{slope})}$] and cycle threshold ($C_T$) values for the two amplicons (chromosome, *c*, and plasmid, *p*) (33). The PCN of pLSEIA was estimated at 2.8 ± 1.4 (mean ± standard deviation) plasmid copies per chromosome (from 3 independent DNA extracts). Taking into account the respective sizes of pLSEIA (29 kbp) and the *L. casei* chromosome (2.9 Mbp), the theoretical percentage of pVI110 nonpreferential integration in pLSEIA should be between 1 and 5%.

To confirm the diversity of mutants and to identify the nature of the target sequences of the pVI110 transposon, the randomly selected mutants were identified by genomic DNA sequencing with primers OLB215 and OLB221 (Table 2), which target the transposon sequence extremities (Fig. 1C and Table 3). In regard to *L. delbrueckii* subsp. *bulgaricus*, more than 80% ($n = 17$) of sequenced targets were located in intergenic regions (IGR). Although four mutants were obtained in the IGR Ldb2086/Ldb2087, the target sequences of the pVI110 insertions were different, suggesting that this region is most likely not a hot spot of integration. Noticeably, target sites are surrounded by inverted repeats predicted to form hairpins with $\Delta G < -9$ Kcal (calculated with Oligo Analyser freeware). Alignment of pVI110 target sequences revealed a preferential insertion in A/T-rich regions, as seen for other mobile elements, like Tn*1545* in *Clostridium* and *Listeria* (5, 8), and several insertion sequences (11, 28). The nucleotide sequences of pVI110-target junctions in *L. delbrueckii* subsp. *bulgaricus* also revealed a 3-bp (occasionally 4-bp) duplication generated upon integration. Analysis of the target sequences suggests that triplets C/A A/T T/A may be preferential target sites for pVI110 in *L. delbrueckii* subsp. *bulgaricus*. Of the 20 random pVI110 transposon targets sequenced for *L. casei* (Table 3), 50% ($n = 10$) were located in intergenic regions, while the *L. casei* genome contains about 20% noncoding regions (7). Ten percent ($n = 2$) of mutants correspond to two different integration sites of pVI110 in pLSEIA, which represents only twice the maximal theoretical rate. This result reveals that pLSEIA is not a significant preferential host for pVI110 integration, indicating that the presence of the pLSEIA plasmid in LC334 is not an obstacle to obtaining saturated mutagenesis libraries. In contrast to the results for *L. delbrueckii* subsp. *bulgaricus*, only 20% of the *L. casei* target sites were located in inverted repeats predicted to form hairpins. Moreover, no preferential insertion in A/T-rich regions was observed. Despite the general, presumed random insertion of most transposons, many of them show a target preference (for reviews, see references 11 and 28). This targeting could in fact be a result of selective means to avoid affecting host fitness and, consequently, to promote transposon dissemination. Since *L. delbrueckii* subsp. *bulgaricus* is closely related to *L. johnsonii*, the original host of IS*1223* (39), IS*1223* is likely to preferentially target noncoding sequences to preserve its host genome. Interestingly, this bias is reduced in *L. casei*, which is phylogenetically more distant from *L. johnsonii* (3, 40), and is reduced even further in *E. coli*, suggesting that insertion sequences may display a more random integration in phylogenetically distant bacteria.

In conclusion, this work describes the use of an IS*3*-like transposition mechanism to engineer a novel transposition system based on the P_{junc}-TpaseIS$_{1223}$ two-plasmid system for Gram-positive bacteria. Our results demonstrate that this system is functional in *L. delbrueckii* subsp. *bulgaricus* and *L. casei* and produces a high rate of stable integrants (at least 10,000 mutants per transformation for *L. casei*) despite the relatively poor transformation rate of lactobacilli. This system presents the advantage of promoting transposition of a suicide plasmid which contains P_{junc} (pVI110) provided in *trans* with a helper plasmid (pVI129) supplying TpaseIS$_{1223}$. Thanks to this design, no sibling clones from early transposition events (31) can appear, and as pVI110 is stably produced in *E. coli*, it can be easily manipulated by inserting a reporter gene or used for signature-tagged mutagenesis. In view of the efficient transposition activity observed in the species tested (e.g., *Bacillus subtilis*, *Lactococcus lactis*, *Lactobacillus plantarum*, and *Enterococcus faecalis*; unpublished results), this transposition system may have a broad application in Gram-positive bacteria, particularly in LAB.

ACKNOWLEDGMENTS

We thank P. Polard for stimulating discussions on transposition mechanisms which led us to initiate this work. We also thank S. Kulakauskas for sharing data on *L. lactis*. We are grateful to Ellen Arena for the English revision of the manuscript.

This work was supported by ERC Advanced grant HOMEOEPITH.

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